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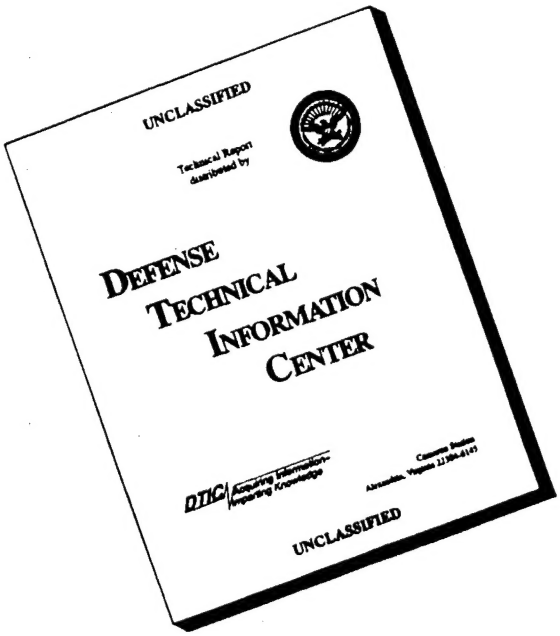

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Rabbit tracheal epithelial cells showed time-dependement mucin gene expression and secretion when cultured in a serum-free and hormone-supplemented medium with retinoids (vitamin A). In the absence of vitamin A, the cells showed squamous differentiation with reduced mucin message. When supplemented with retinoids, the cells reverted back to normal. Time-dependent (5-20 minutes) exposure of rabbit tracheal explants to smoke from pine wood resulted in inflammation of the epithelium with very high expression of mucin message. When cultured in a medium with retinoids, the epithelium became normal with normal mucin gene expression. Without retinoids in the culture medium, injury to epithelium and mucin message did not recover well. Addition of an antisense mucin oligomer resulted in partial inhibition of mucin message and the cells showed normal profile. Thus, combined therapy of retinoids and antisense mucin oligomer have clinical implication in the treatment of people, including combat soldiers, exposed to toxic exposure like smoke.

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Introduction

Inhalation of smoke and combustion products from fire-related aaccidents associated with military activities as well as amongst general public results in acute lung injury associated with excessive secretion of mucus in the tracheobronchial and parenchymal regions of the lung, the injury that has been suggested to be one of the major contributing factors in the morbidity and mortality of patients (1-6). Transient pulmonary (both laryngeal and tracheobronchial) edema is one of the pathophysiological events of this injury, together with blockage of bronchial tubes by both mucus and slough segments of the epithelium. Changes in the differentiation of ciliated and nonciliated tracheal epithelial cells have also been noted in smoke-related injuries and basal cells have been implicated in the repair process (7-9). However, the mechanism involved in the differentiation of tracheal epitheilial cells as well as excessive secretion of mucus and the factors involving these processes after injuries have not been thoroughly examined. This project is directed towards determining the effect of smoke inhalation and combustion products - singly and in combination - on the synthesis and secretion of mucins (components of mucus) and finding the role of retinoids (vitamin A), antisense mucin oligomer and other respiratory drugs in preventing excessive secretion of mucus as well as repair of injury to tracheal-bronchial epithelium. The ultimate goal is to prevent disease recurrence and control the amount of secretion caused by the offending exposure.

For several years, we at DCI, WBAMC, have been engaged in studying the effect of retinoids, different pharmacologic agents and antisense mucin oligomers on the mucin gene expression and secretion of mucins in organ as well as in isolated tracheal epithelial cells cultured in a serum-free and hormone-supplemented medium. To date, we have found that retinoids are required for normal function of tracheal epithelial cells when grown in a serum-free and hormone-supplemented medium. Without retinoic acid, the cells neither expressed mucin message nor maintained normal cytological appearance (10-13). When retinoids were added back to the culture medium, the cells grew normally and the mucin message was expressed again. Addition of a 20-mer antisense mucin oligomer blocked the expression of mucin message as well as mucin secretion while keeping the cell profile normal (12). It was also found that of all the pharmalogicals used, only steroid, prednisolone, had a marked inhibitory effect on the mucin message (11). Squamous differentiation of epithelial cells which occurred in the absence of retinoids in the culture medium was reversed by supplementing the medium with retinoic acid. Thus, the combined therapy of retinoids and antisense mucin oligomers has the potential in clinical application of repairing injury and inhibiting mucin oversecretion in tracheobronchial epithelium of people, including combat soldiers, exposed to toxic substances like smoke. The examination of the effect of smoke exposure on the tracheal epithelium has begun in our laboratory and the progress of those experiments is dicussed below (body).

Body

a. **Effect of retinoids**

In preliminary experiments prior to smoke exposure, we cultured isolated rabbit tracheal epithelial cells in a serum-free and hormone-supplemented medium (10) with and without retinoic acid to determine the extent of control of mucin gene expression and secretion by these agents. The cells showed time-dependent mucin gene expression when cultured in the medium with retinoic acid. Without retinoic acid, however, the cells did not express mucin gene and this inhibition was associated with squamous differentiation of the cells as observed by electron-microscopy. The retinoid-induced mucin gene expression and secretion was inhibited by a 20-mer mucin antisense oligomer and the cells reverted back to the normal condition. This initial observation led us to the conclusion that combined retinoids and antisense treatment may offer an alternate approach in the management of mucus hypersecretion as well as maintenance of normal tracheal epithelium exposed to offending exposure like smoke. These results will appear in the journal INFLAMMATION in august, 1995 (reference 13, press copy enclosed).

We have observed similar, if not identical, phenomena using rat tracheal explants in cculture (11,12).

b. **Smoke exposure**

After assembling the smoke exposure system, we have been able to get interesting results which are described below.

Whole rabbit trachea was opened up in the middle and suspended in minimum essential medium (MEM) and exposed to whole smoke by burning pine wood (40 g) in a combustion chamber with bellows blowing the smoke to the exposure system. After exposure for different periods of time (5-30 minutes), the trachea was cut into rings and placed in a serum-free and hormone-supplemented medium (10-13) with and without retinoic acid and the incubation continued for several hours. The exposure time of smoke was limited to 20 minutes, because exposure longer than this period resulted in severe loss of the cells as well as acute damage to the epithelium which could not recovered by culture condition employed in this study.

Morphological studies by electron microscopy of the trachea before and after exposure to smoke (20 minutes) indicated inflammation in the exposed trachea with numerous mucus secretory granules (Figure 1B) which were barely present in the normal cells (Figure 1A). After culture of the explants for 96 hours, the morphology of the cells showed normal features in presence of retinoids (Figure 1C). The cells which were cultured without retinoic acid showed signs of deprivation associated with retardation of growth (Figure 1D). This change in cell morphology was associated with change in mucin gene expression as measured by hybridizational analysis using an antisense mucin probe. After exposure to smoke , the mucin gene expression was increased several fold. After incubation for 96 hours in culture medium with retinoic acid, the cells showed reduced mucin gene expression. Without retinoic acid in the culture medium, there was hardly any mucin message. Addition of mucin antisense oligomer to the culture exposed to smoke also resulted in the reduction of mucin message. The results suggest that retinoids have a

strong influence in maintaining healthy tracheal epuithelium even after exposure to smoke.

Conclusions

In conclusion, rabbit tracheal explant, after exposure to smoke, showed inflammation in the epithelium with changes in the ultrastructure as well as in the strong expression of mucin gene. Both retinoic acid and an antisense mucin oligomer were found to inhibit mucin gene, at the same time keeping the tracheal epithelium normal. Thus, combined therapy of retinoids and antisense oligomer may have some clinical implication in repairing injury as well as inhibition of mucus hypersecretion in tracheo-bronchial epithelium of people, like combat soldiers as well as general public, exposed to toxic substances like smoke.

The followings are the future plans which will be pursued to understand the mechanism involved in the control of mucus hypersecretion as well as repairing injury in the trachea exposed to smoke. (a) Study time- and dose-dependent mucin mRNA expression and secretion in rabbit tracheal explants and isolated cells exposed to smoke and smoke-related particles from burning pine wood and cotton and the effect of retinoids and antisense oligimer on this system. Changes in the morphology will be studied by ultramicroscopy. The use of isolated cell culture will enable us to determine more accurately the involvement of these cells in the repair process. (b) Study and compare the effect of antisense therapy versus other pharmacologic agents, such as steroids, on mucin mRNA level and secretion as well as cell differentiation and proliferation of the tracheal culture exposed to smoke as stated above. Also, examine the stability of mucin message. (c) If possible, produce an immortal mucin producing tracheal cell line by infecting with different nonpathogenic viruses. Examine the effect of long-term exposure of smoke on these cells and the process of recovery of injury and inhibition of oversecretion by retinoids and antisense oligomers.

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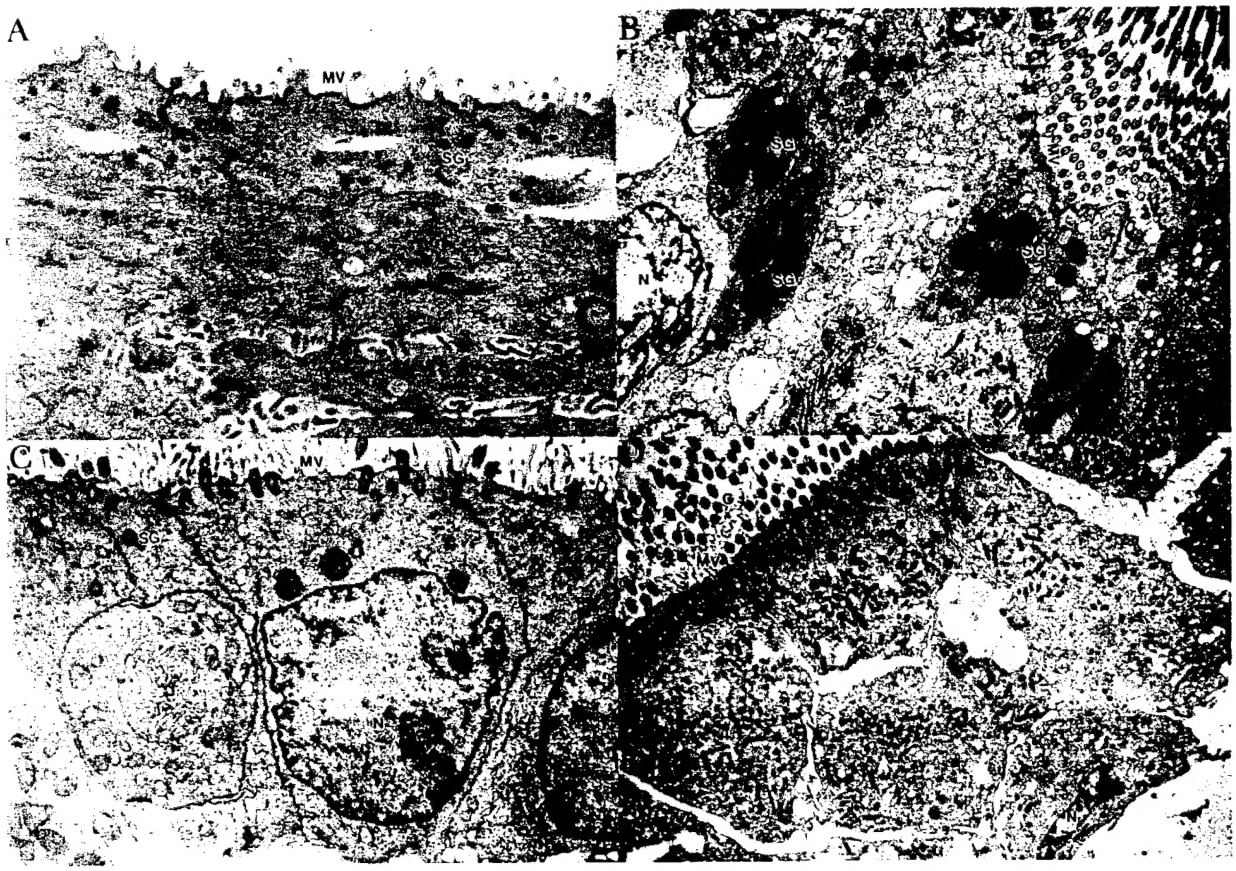

Figure 1. Ultrastructure of rabbit tracheal explant before and after exposure to smoke and the effect of retinoids on this system. A. Normal rabbit trachea. B. Tracheal explant after exposure to smoke from pine wood for 20 minutes. C. +retinoic acid, 96 hours of culture. D. -retinoic acid, 96 hours of culture. MV: microvilli; SG: secretory granules; N, nucleus.

Appendix

A major preliminary work on this project has been accepted for publication in the journal INFLAMMATION and will appear in august, 1995. A copy of the paper is enclosed.

Part of this study was presented to the annual meeting of Society of Glycobiology at University of Notre Dame, Notre Dame , IN 46556, November 9-12, 1994 and published in the Journal, Glycobiology, Vol.4, 1994 . A copy of the abstract is enclosed.

Inflammation, Vol. 19, No. 4, 1995

RETINOIC ACID-REGULATED CELLULAR DIFFERENTIATION AND MUCIN GENE EXPRESSION IN ISOLATED RABBIT TRACHEAL EPITHELIAL CELLS IN CULTURE[1]

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Abstract—Rabbit tracheal epithelial cells were cultured in a serum-free and hormone-supplemented medium with and without retinoic acid. The cells showed time-dependent mucin gene expression when cultured in the medium with retinoic acid. In the absence of retinoic acid, however, mucin mRNA was barely detectable in the cells. When retinoic acid was added back to the medium, the mucin message was prominent again. Actinomycin D and cycloheximide did not inhibit mucin gene expression. The mucin message was slightly elevated by cAMP agonists. A mucin antisense oligomer inhibited the retinoic acid-induced mucin mRNA expression and secretion, thus offering an alternate approach in the management of mucus hypersecretion in upper airway respiratory diseases such as chronic bronchitis, asthma, and cystic fibrosis.

INTRODUCTION

Bronchial mucus glycoproteins (mucins) are localized on the apical surface of the mucus membrane, where they function in the mucociliator–escalator systems that protect the airways from microscopic particles and infections. Excessive accumulation of mucus is one of the major contributors to airway obstruction in people suffering from upper airway respiratory diseases, such as asthma, chronic bronchitis, and cystic fibrosis. The precise mechanism(s) involved in the accumulation of mucus in these diseases is not known, but all evidence

[1]The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Presented in part at the 23rd Annual Meeting (November 9–12, 1994) of the Society for Glycobiology, University of Notre Dame, Indiana.

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points to overproduction rather than defective clearance by the mucocilary systems.

It has been reported (1–3) that tracheal epithelium can be maintained in a serum-free and hormone-supplemented medium containing retinoids (vitamin A) for several weeks; cultured cells were found to secrete mucins into the medium, as measured by the incorporation of radiolabeled sugars. Previously, we observed (4) that rabbit tracheal epithelial cells, grown in a serum-free and hormone-supplemented medium, expressed mucin gene when total RNA isolated from the cells was hybridized with a 30-mer oligonucleotide probe derived from a rat intenstine peptide tandem repeat sequence, TTTPDVTTTP, RMUC 176 (5). However, in the absence of retinoic acid in the culture medium, the cells did not grow or produce mucin. Recently, we observed (6, 7) that the expression of mucin mRNA in rat tracheal explants at various times of growth in a serum-free and hormone-supplemented medium was strongly induced by retinoic acid. In the absence of retinoic acid, the mucin message in he tracheal organ culture decreased considerably. Addition of retinoic acid to the deficient medium restored the mucin message back to normal. Addition of various pharmacologic agents also had some marginal effect on mucin mRNA expression in rat tracheal organ culture grown with and without retinoic acid. However, the effect of these reagents on mucin mRNA expression in an isolated tracheal epithelial cell culture system has not been reported.

Recently, there has been intense interest in developing antisense agents for sequence-specific inhibition of gene expression (7–10) and using them as therapeutic agents. Thus, viral as well as cancer message expression has been successfully inhibited by antisense oligomers. In most of these experiments, the antisense oligomer has been chemically modified, and the most frequent use was that of phosphorothioate derivative. The same derivative of an antisense oligomer has recently been used in the treatment of acute myeloblastic leukemia (11).

In view of the above findings, we have examined the control as well as stability of mucin mRNA expression in isolated rabbit tracheal epithelial cells in culture in a serum-free and hormone-supplemented medium with and without retinoic acid and the effect of an antisense oligodeoxynucleotide in inhibiting this retinoic acid-induced mucin gene expression.

MATERIALS AND METHODS

All chemicals were of the highest quality available and obtained from various sources as described before (1, 4, 6, 7).

Cell Culture

Airway epithelial cells were isolated from rabbit trachea (New Zealand white rabbit, Hazelton Research Laboratories, Denver, Pennsylvania), and cultured on collagen-coated dishes in a serum-free and hormone-supplemented medium according to the procedures described previously (1, 4, 6, 7). Cells were incubated at 37°C in a humidifed incubator containing air–CO_2 (19:1), and the medium was changed every two days. The epithelial nature of the cells containing secretory granules, microvilli, tonofilaments, and desmosomes has been established in our laboratory by electron microscopy (4).

Isolation of RNA

Total RNA was isolated from cells (2.0×10^6) using Clontech's RNA isolation kit, which utilizes a guanidium thiocyanate–phenol–chloroform single-step extraction procedure, as described previously (4, 6, 7, 12). The extracted RNA was precipitated with 3 M sodium acetate and absolute alcohol and kept at −70°C until needed.

Northern and Dot Blot Hybridization

RNA was collected by centrifugation at 5000*g*, dried under vacuum, and rehydrated with a minimum volume of TE buffer and RNAase inhibitor, as described before (4, 6, 7). An aliquot of RNA preparation (5–10 μg) was run on a formaldehyde–agarose (1%) gel in 1 × Mops buffer (6). The gel was stained with ethidium bromide. RNA was transferred to hybond membrane from the gel for Northern hybridization. The membrane was dried under vacuum at 80°C for 2 h.

A direct transfer of RNA (0.5–1.5 μg) to spots on the Hybond membrane was made in a dot blot apparatus and the membrane was dried as described above. The membrane was hybridized with an E-linked antisense 30-mer oligonucleotide to rat intestine mucin protein tandem repeat sequence, RMUC 176, TTTPDVTTTP, (5'-X AGG GGT GGT GGT CAC ATC AGG AGT GGT GGT-3', X = amine) (5)—this procedure has been described in detail elsewhere (4, 6, 7). After hybridization, the membrane was stained with Lumi-Phos solution, placed between two acetate sheets, and exposed to Kodak XAR-5 film for 3 h at 37°C. All experiments were performed with the same concentration (1.0 nM) of the probe under identical conditions.

The membrane was also hybridized with an E-linked antisense 30-base oligonucleotide to mouse type I keratin protein sequence, GGDQSSKGPR (5'-X TGG TCC TTT AGA TGA TTG GTC GCC GCC ACC-3', X = amine) (13), as described before (6, 7).

The following phosphorothioate derivatives of oligonucleotides were used in these studies: sense oligo—5'-ACCACCACTCCTGACGTC-3' to rat intestine mucin cDNA sequence (5) (control); and antisense oligo—5'-AGGGGTGGTGGTCACATC-3' to rat intestine mucin cDNA sequence (5).

Effect of Retinoic Acid, Actinomycin D, Cycloheximide, cAMP Agonists, and Antisense Oligomer

Retinoic Acid. Time-dependent expression of the mucin message with and without retinoic acid (0.1 μM) was investigated by incubating the rabbit tracheal epithelial cells (2.0×10^6) in 3.0–4.0 ml of culture medium for 0, 48, 96, 120, and 144 h under the conditions described before (4, 6, 7). In order to determine the effect of retinoic acid on mucin mRNA content in the cells grown in medium without retinoic acid, the cells were initially maintained in a retinoic acid-deficient medium for 96 h before addition of this reagent, and the incubation continued for another 96 and 144 h. Duplicates and controls were run under the same conditions.

Actinomycin D and Cycloheximide. The effect of actinomycin D (20 μg/ml) and cycloheximide (20 μg/ml) on the expression of the mucin message in these cells grown in the presence of retinoic acid was examined by adding these reagents at 96 h of growth and continuing incubation for additional 12-, 24-, 48-, and 96-h periods of time. Duplicates and controls were run under the same conditions.

cAMP Agonists. In order to determine the effect of cAMP agonists, forskolin (100 μM), PGE_2 (100 μM), bt_2cAMP (100 μM), and the calcium ionophore A23187 (100 μM), on mucin gene xpression in these cells, the cells were grown for 96 h before addition of these reagents to the medium and the incubation continued for another 48 h. Duplicates and controls were run under the same conditions.

Sense and Antisense Oligonucleotides. The cells (2.0×10^6) were grown for 120 h before addition of 10 μCi of [6-^{3}H]glucosamine (4), sense and antisense oligomers (0.53 nmol/ml), and the incubation continued for another 48 h. After the reaction, the cells were removed from the medium by centrifugation. Duplicates were run under the same conditions.

Total RNA was isolated from the cells and hybridized with the respective oligonucleotide probe, as described above. The density of the signal was measured by the BioRad densitometer.

The medium was dialyzed against water at 4°C overnight with two changes and lyophilized. The lyophilized material was digested with hyaluronidase and analyzed for mucin by Sepharose 2B chromatography and gel electrophoresis as described in detail elsewhere (4). Radioactivity was determined in a Beckman LS 3801 counter.

Phase-Contrast and Electron Microscopy.

The cells at different stages of growth were examined with a phase-contrast microscope.

The cells prepared for electron microscopy were fixed in 3.5% (w/v) glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections of the sample were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (4, 6, 7).

RESULTS

The changes in the morphology of rabbit tracheal epithelial cells were examined by phase-contrast microscopy at 48, 96, and 144 h of growth in a medium with and without retinoic acid (Figure 1 A–F). As is seen in this figure

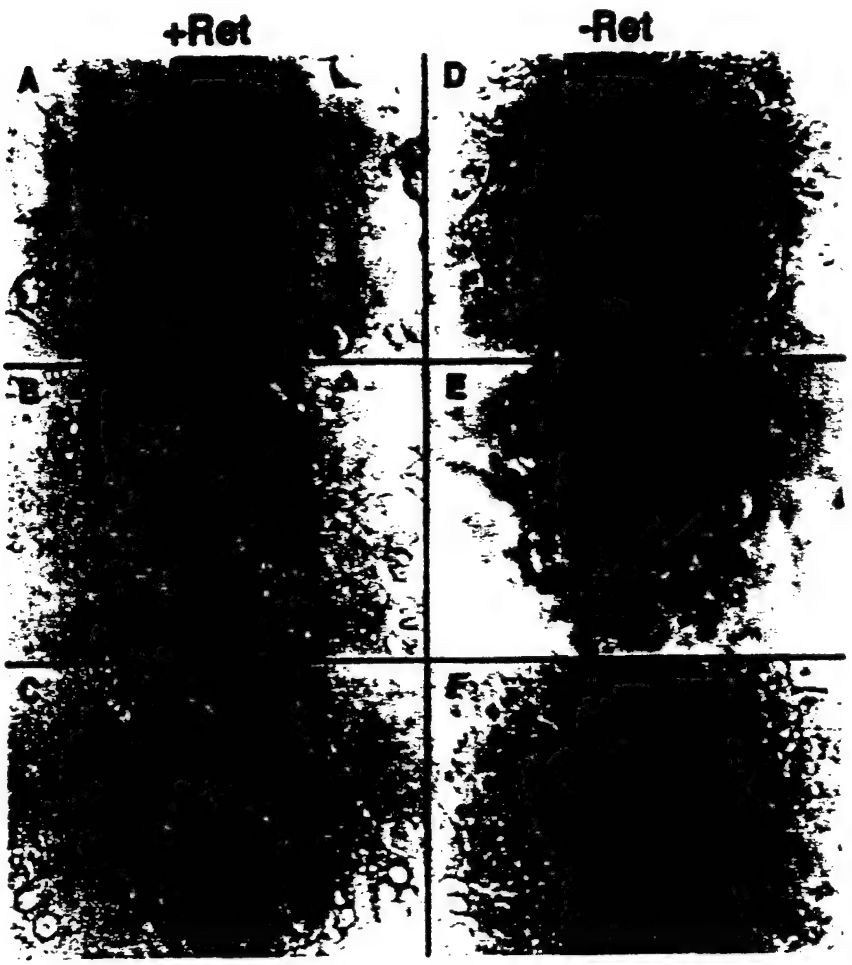

Fig. 1. Phase-contrast micrograph of rabbit tracheal epithelial cells at 48, 96, and 144 h of growth in a serum-free and hormone-supplemented culture medium with and without retinoic acid. The cells were cultured as described in Materials and Methods. A, B, C: +retinoic acid; D, E, F: −retinoic acid. Magnification ×160.

and as also observed by several investigators in tracheal epithelial cells from other animals (14–16), the difference in morphology of the cells grown with and without the presence of retinoic acid in the medium is quite striking. Retinoic acid-sufficient cells had a tendency to form monolayers with the passage of time, whereas deficient cells showed changes towards squamous differentiation. At a later stage of growth, the retinoic acid-deficient cells tend to detach themselves

Fig. 2. Transmission electron microscopy of rabbit tracheal epithelial cells grown with and without retinoic acid (A) (+retinoic acid): SG, secretory granules; MV, microvilli; arrow, desmosomes. (B) (−retinoic acid): arrow, desmosomes. Note the stratified epithelial layer of the cells grown without retinoic acid and absence of secretory granules. Magnification ×13,000.

from the collagen layer. Transmission electron microscopic studies of the cells at 144 h of growth in a medium containing retinoic acid indicated that the cells contained tonofilament bundles and were joined by desmosomes. Microvilli and occassional secretory granules were also observed (Figure 2A). In the absence of retinoic acid, however, the epithelial layers were stratified with development of squamous metaplasia. No secretory granules were observed (Figure 2B). This change in cell morphology was associated with a change in mucin gene expression. Dot blot hybridization using an E-linked rat intestine mucin tandem repeat

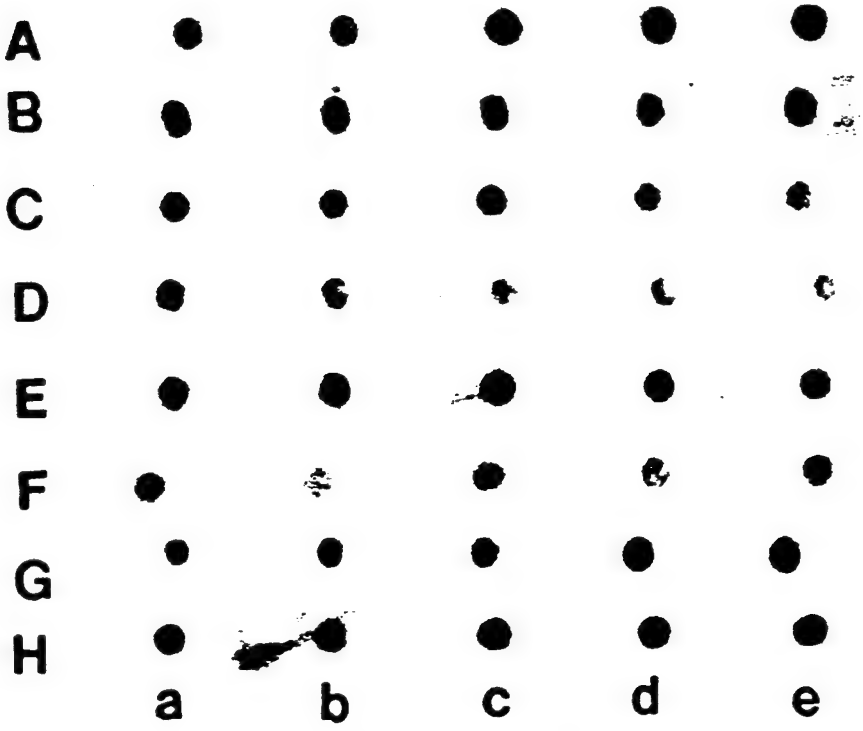

Fig. 3. Dot blot hybridization of total RNA (1.2 μg) from rabbit tracheal epithelial cells at different times of growth in a serum-free and hormone-supplemented medium with and without retinoic acid. Hybridization with E-linked rat intestine mucin probe was performed as described in the experimental section. (A) a–e (mucin probe), total RNA from culture (+retinoic acid) at 0, 48, 96, 120, and 144 h of growth; (B) a–e, same as A except hybridized with β-actin probe; (C) a–e, same as A except hybridized with type I keratin probe; (D) a–e (mucin probe), total RNA from culture (−retinoic acid) at 0, 48, 96, 120, and 144 h of growth; (E) a–e, same as D except hybridized with β-actin probe; (F) a–e, same as D except hybridized with type I keratin probe; (G) a–e, same as D except that retinoic acid was added to the medium at 96 h of growth (c) and the culture was allowed to grow for another 96 and 144 h (d, e); (H) a–e, same as G except hybridized with β-actin probe.

probe, RMUC 176 (5), to total RNA from the cells grown at different periods of time showed that the mucin message, after expressing at a lower level at 0 and 48 h (Figure 3A, a–b), showed a stronger signal from 96 h to all subsequent time points (Figure 3A, c–e). The expression of β-actin mRNA in these preparations is shown in Figure 3B, a–e. Type I keratin message showed a positive signal in all the preparations (Figure 3C, a–e). In the absence of retinoic acid, the mucin message was considerably reduced with the progression of time (Figure 3D, a–e), whereas the expression of β-actin mRNA remained steady (Figure 3E, a–e). The hybridizational signal for type I keratin message was evident in these preparations (Figure 3F, a–e). Addition of retinoic acid to the latter culture at 96 h of growth resulted in the recovery of mucin message within 96 h of additional incubation (Figure 3G, c–e). The level of β-actin message in these preparations is shown in Figure 3H, a–e.

Addition of actinomycin D (20 μg/ml), an inhibitor of transcription, to the culture at 96 h of growth in the medium containing retinoic acid did not result

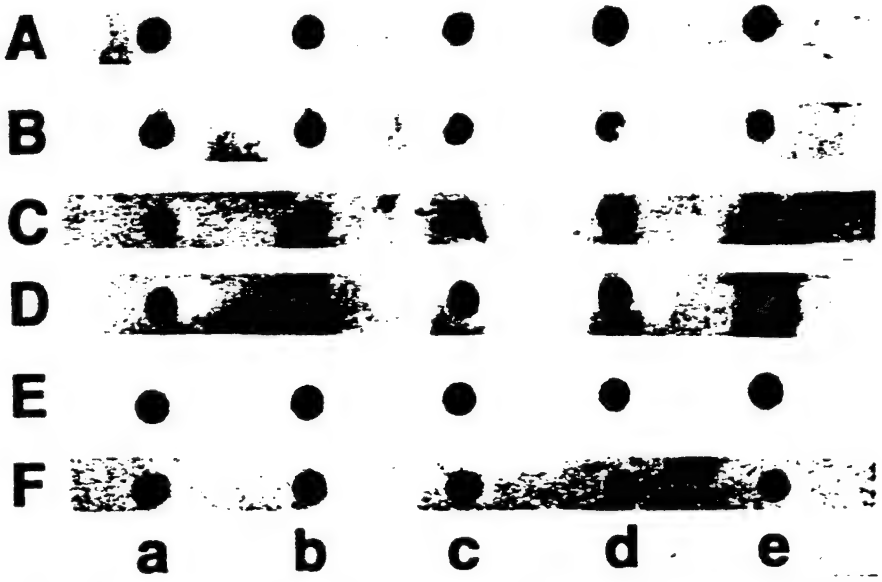

Fig. 4. Effect of actinomycin D (20 μg/ml), cycloheximide (20 μg/ml), cAMP agonists (100 μM), and calcium ionophore A23187 (100 μM) on mucin mRNA from rabbit tracheal epithelial cells grown in a medium with retinoic acid. The cells were grown for 96 h (control) before addition of the reagents, and the incubation continued for the specified period of time. Total RNA (1.5 μg) was hybridized with E-linked rat intestine mucin probe as described in the experimental section. (A) a–e (actinomycin D), control, 12, 24, 48, and 96 h; (B) same as A except hybridized with β-actin probe; (C) a–e (cycloheximide), control, 12, 24, 48, and 96 h. (D) a–e, same as C except hybridized with β-actin probe; (E) a (control), b (forskoline), c (PGE_2), d (bt_2cAMP), and e (calcium ionophore A23187); (F) a–e, same as E except hybridized with β-actin probe.

in the reduction of the mucin message (Figure 4A, a–e) and was similar to the pattern shown in Figure 3A, a–e, whereas β-actin mRNA expression decreased considerably (Figure 4B, a–e). The inhibitor of translation, cycloheximide (20 μg/ml), slightly increased the mucin message (Figure 4C, a–e). The level of β-actin mRNA in these preparations is shown in Figure 4D, a–e. Incubation of the cells at 96 h of growth with activators of the cAMP pathway, forskoline, PGE_2, and bt_2cAMP, and calcium ionophore A23187 resulted in a slight increase in the expression of the mucin message (Figure 4E, a–e). The expression of β-actin message in these preparations is shown in Figure 4F, a–e.

Agarose gel (1%) electrophoresis of the total RNA from rabbit tracheal epithelial cells at 144 h of growth showed a typical gel profile of 28S and 18S RNA in the preparation (Figure 5A). Northern hybridization of the RNA prep-

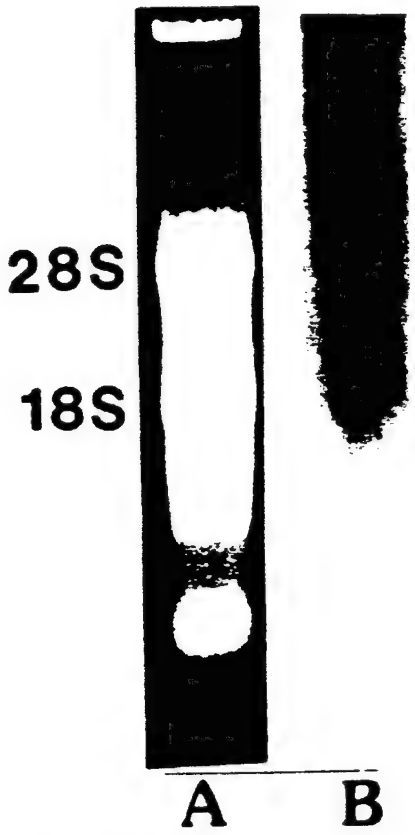

Fig. 5. (A) formaldehyde–agarose gel (1%) electrophoresis of total RNA (10 μg) from rabbit tracheal epithelial cells at 144 h of growth in a serum-free and hormone-supplemented medium with retinoic acid. The gel was stained with ethidium bromide. (B) Northern hybridization of total RNA after transfer to the hybond membrane and probed with E-linked mucin probe.

Fig. 6. Transmission electron microscopy of rabbit tracheal epithelial cells treated with sense (A) and antisense (B) oligonucleotides. The cells were grown in medium with retinoic acid as described in the methods section. (A) MV, microvilli; SG, secretory granules; arrow, junctional complexes; N, nucleus. Magnification ×14,000. (B) MV, microvilli; arrow, desmosome. No secretory granules were noted. Magnification ×13,000. (C) a–b, dot blot hybridization of total RNA (1.5 μg) from cells treated with sense (a) and antisense (b); (D) a–b, same as C except hybridized with β-actin probe.

aration resulted in a diffuse pattern with a molecular weight ranging from 2.0 kb to >9.5 kb (Figure 5B). Such a polydisperse signal of mucin mRNA on Northern analysis has been observed by others before (17-20). The reason for this polydispersion is not known.

Transmission electron microscopy showed that the cells grown in medium containing retinoic acid and the phosphorothioate derivative of the sense oligomer were covered with surface microvilli and joined by small desmosomes and apical junctional complexes (Figure 6A). In addition to numerous secretory granules, the cytoplasm contained Golgi complexes, mitochondria, and segments of rought endoplasmic reticulum. In the presence of antisense oligomer, the cells displayed surface microvilli and small desmosomes, but no secretory granules (Figure 6B). This change in cell morphology was associated with a change in mucin gene expression and secretion in these cells. Addition of the antisense oligomer to the cells resulted in an approximate 2.3-fold decrease in the expression of mucin mRNA when compared to the effect of the sense (control) oligomer (spot density = sense, 9.35: antisense, 4.15) (Figure 6C, a–b). Figure 6D, a–b indicates the β-actin message expression in these preparations. The decrease in the expression of mucin mRNA was followed by 2.4-fold drop in the secretion of mucin into the medium [radioactivity (cpm/ml): sense, 8940, antisense, 3754).

DISCUSSION

The present study, demonstrating that retinoic acid stimulated the growth of isolated rabbit tracheal epithelial cells in a serum-free and hormone-supplemented culture medium and caused up-regulation of mucin message, is an extension of our previous studies (6, 7), in which we noted that retinoic acid was also required for optimal mucin gene expression in rat tracheal explants grown in the same culture medium. In the absence of retinoic acid, rabbit tracheal epithelial cells did not grow normally, nor did they express mucin gene strongly. Instead, the entire epithelum was stratified with an additional change towards squamous metaplasia. However, the mucin message recovered when retinoic acid was reintroduced to the retinoic acid-deficient medium. Thus, the mucin gene in rabbit tracheal epithelial cells in culture seems to be transcriptionally activated by retinoic acid, and the mucin mRNA expression is strongly associated with differentiation and proliferation of epithelial cells.

The results presented in this report and in our earlier studies (4, 6, 7) regarding the effect of retinoic acid in the enhancement of mucin gene expression are in agreement with those of Jany and Basbaum (21), who suggested that induction of the mucin message is the primary event that leads to increased

synthesis and secretion of mucins in rat trachea exposed to SO_2. Retinoid-dependent gene regulation has been observed previously in squamous differentiation of tracheal epithelial cells (22).

The persistence of mucin mRNA at high levels in the presence of the transcription inhibitor, actinomycin D, indicates that mucin transcripts in these cells are highly stable molecules. The inhibitor of translation, cycloheximide, did not block the on-going protein synthesis in the cells. Stabilizations of neurofilament mRNA in primary sensory neurons, vitellogenin mRNA by estrogen in primary liver cells, and transferrin receptor mRNA by chelation of iron have been found to occur in the presence of both actinomycin D and cycloheximide. It has been suggested that the process could be mediated by short-lived factor(s) that protects the transcripts from destabilization due to the presence of hormones or ligands (23–25). Such a mechanism involving the retinoic acid receptor complex or a cascade of regulatory proteins in tracheal epithelial cells may exist to protect the mucin message from destabilization. Alternatively, the mucin transcripts could be indirectly stabilized by factor(s) that regulate both cAMP pathway as well as those involved in mucin synthesis (2, 4, 26, 27). However, no effects comparable to those seen in the presence of retinoic acid were observed upon addition of either forskoline, PGE_2, bt_2cAMP, or calcium ionophore A23187 to these primary tracheal epithelial cell lines.

The study as presented here clearly indicates that retinoic acid regulates mucin gene expression in isolated rabbit tracheal epithelial cells cultured in a serum-free and hormone-supplemented medium and that this expression is associated with differentiation and proliferation of these cells. This retinoic acid-induced mucin gene expression and secretion also can be inhibited by mucin antisense oligomer. The use of an antisense oligomer to regulate gene expression is fast becoming an alternative approach for therapeutic intervention into disease processes such as cancer and viral infection (7–11). The present study can be extended further to examine whether antisense therapy can be used in the management of hypersecretion of mucus in upper airway respiratory diseases such as chronic bronchitis, asthma, and cystic fibrosis.

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14.02

Characterization of the Glycoconjugates of Mammalian Bladder Epithelium M.S. Buckley, S. Huber, D.R. Erickson* and V.P. Bhavanandan, Depts. Biochem. & Mol. Biol. and Surgery*, M.S. Hershey Medical Center, Penn State University, Hershey, PA

The mucosal surface of the urinary bladder acts as an effective barrier against invasion by pathogenic microorganisms and injury from toxic substances in the urine. Defects in the bladder mucosa could thus be an important factor in the development of diseases such as bladder cancer, interstitial cystitis (IC) and lower urinary tract infections. The presence of a mucin-type glycoprotein, epitectin, on the urothelium of human bladder has been previously established (Bramwell *et al.*, Br. J. Cancer **48**, 177). In this study, anti-epitectin antibody was found also to stain rabbit bladder mucosa. Rabbit bladder explants cultured in the presence of [^{3}H] glucosamine and unlabeled bladders were used to isolate the glycoconjugates. Gel filtration, treatment with hyaluronidase, ion exchange chromatography and CsTFA density gradient centrifugation yielded high molecular weight sialoglycoproteins which are being characterized. Immunohistochemical examination was performed on bladder epithelial biopsy specimens from normal individuals and IC patients. Anti-heparan sulfate antibody reacted strongly with all layers of the bladder wall and there was no noticeable difference in the level of staining of patient and control specimens. In contrast, with anti-epitectin antibody staining was limited to the epithelium. Preliminary indications are that biopsy specimens from IC patients had weaker and more patchy staining compared to specimens from non-IC controls. (Supported by NIH grant DK-47511).

14.03

Characterization of KDN-Containing Mucin from the Skin Mucus of the Loach, *Misgurnus anguillicaudatus*

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Fish skin is coated with mucus containing a variety of mucus cell secretions. We report the isolation and characterization of 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN)-containing mucin from the skin mucus of the loach, *M. anguillicaudatus*.

Presence of KDN in loach skin mucus — Dried loach skin mucus was treated with 0.1 N formic acid at 80°C for 1 h. The hydrolyzate was passed through an AG50-X8(H^+), followed by AG1-X8(formate) chromatography. A thiobarbituric acid-positive compound was eluted from the column with 0.5 N formic acid. The compound was characterized to be KDN by FAB-MS and 500 MHz ^{1}H-NMR spectroscopy.

Characterization of KDN-containing mucin — Mucin was isolated from dried loach skin mucus by DE-52 chromatography, nuclease digestion and Sepharose CL-4B gel filtration. The purified mucin was composed of KDN (38.5%), GalNAc (24.6%), Gal (3.3%), NeuAc (0.4%) and protein (rich in Thr). The mucin (100 mg) was treated with alkaline borohydride to release oligosaccharide alditols. The oligosaccharide alditols were separated by Sephadex G-25 gel filtration to obtain two major oligosaccharide alditols, I (15.4 mg) and II (15.6 mg), and a glycopeptide fraction eluted at the void volume. I and II accounted for 46% and 34%, respectively, of all the sugar chains in the mucin. By methylation analysis, I was determined to be KDNα2→6GalNAc-ol and II, KDNα2→3(KDNα2→6)GalNAc-ol. (Supported in part by NIH Grant NS09626).

****14.04

RETINOIC ACID REGULATED MUCIN GENE EXPRESSION IN ISOLATED RABBIT TRACHEAL EPITHELIAL CELLS IN CULTURE

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Rabbit tracheal epithelial cells were cultured in a serum-free and hormone- supplemented medium with or without retinoic acid. The cells showed time-dependent mucin gene expression when cultured in the medium with retinoic acid. In the absence of retinoic acid, the mucin mRNA was barely detectable in the cells. When retinoic acid was added back to the medium, the mucin message was prominent again. Actinomycin D had no effect on mucin mRNA in cultures grown with retinoic acid. The usual precipitous drop in mucin mRNA in cultures lacking retinoic acid was prevented by actinomycin D. Cycloheximide had also no effect on mucin mRNA in retinoic acid sufficient cultures, but, like actinomycin D, it prevented the sharp drop in mucin mRNA in retinoic acid deficient cultures. cAMP agonists had some marginal effects on the mucin mRNA, but none as dramatic as those noted by actinomycin D and cycloheximide in the retinoic acid deficient cultures. A mucin antisense oligomer inhibited the mucin mRNA expression and secretion. The results from our studies suggest that differentiation and hence the induction of mucin gene expression in rabbit tracheal epithelial cells by retinoic acid contribute to regulation of the synthesis of these important components. These findings have important implications in various respiratory diseases, such as asthma, cystic fibrosis and chronic bronchitis, which are characterized by oversecretion of mucus.

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14.05

Isolation and Partial Characterization of Lorenzan Sulfate Glycoproteins (LSGPs) from Shark Electroreceptive Organs.

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Sharks, skates, and rays possess specialized sensory organs, the ampullae of Lorenzini, which detect weak, low frequency electric fields. The ampullae are innervated saccules connected to pores on the skin by canals up to several cm. in length. The ampullae and canals are filled with a viscous jelly with approx. the same electrical conductivity as seawater. Doyle (Biochem. J. 103:325,'67) examined papain digests of the whole jelly and identified a series of sulfated glycopeptides which he termed Lorenzan sulfates. This report describes the purification and partial characterization of mucin glycoproteins from the jelly of hammerhead (HH) and bull sharks. Fractionation of the jelly on SCL-2B in 6 M GuHCl yielded two partially resolved glycoproteins with Kavs of 0.18-0.22 (LSGP-1) and 0.40 (LSGP-2). The major amino acids in all of the LSGPs are Thr (30-34%), Pro (13-16%), and Ile (12%). The monosaccharide composition varies slightly among the gp's with an average molar ratio of Fucose:GalNAc:GlcNAc:Gal 0.4:1.0:0.8:2.0; traces of mannose and glucose are also present. LSGP-1 appears to be a disulfide-linked complex of LSGP-2 subunits since reduction/alkylation of the former shifts its CL-2B elution profile to that of the latter. The major tryptic glycopeptides from reduced/alkylated LSGPs of the bull and HH sharks have Kavs of about 0.60 and 0.45, respectively, on CL-2B. Certain anionic gels undergo dramatic volume changes in the presence of weak electric fields;this may suggest a role for LSGPs in electroreception. (Supported by NSF grant IBN-9396304).

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